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14. ABSTRACT

Intravenous administration of liposomes, including Doxil, can cause severe life threatening hemodynamic changes in pigs. The reaction is due to complement activation, and it is characterized by massive pulmonary hypertension, systemic hypotension and severe cardiac abnormalities including falling cardiac output, tachy- or bradycardia with arrhythmia. There were no data suggesting the involvement of cerebrovascular changes in this reaction, however clinical observations allowed this hypothesis. Here we measured the accompanying changes during liposome infusion by monitoring pulsatile electrical impedance (rheoencephalogram - REG) on the skull (n = 24 pigs, 57 trials, 19 types of liposomes). A transient but significant decrease of REG pulse amplitudes followed the injection of liposomes (78.43 % in the total sample, and 91.66 % in the Doxil subgroup; P = 0.003, n = 12), indicating the involvement of cerebrovascular reaction during liposome infusion.

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Cerebrovascular Involvement in Liposome—Induced Cardiopulmonary Distress in Pigs

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Intravenous administration of liposomes, including Doxil, can cause severe life-threatening hemodynamic changes in pigs. The reaction is due to complement activation, and it is characterized by massive pulmonary hypertension, systemic hypotension, and severe cardiac abnormalities including falling cardiac output, tachy-or bradycardia with arrhythmia. There were no data suggesting the involvement of cerebrovascular changes in this reaction; however, clinical observations allowed this hypothesis. Here we measured the accompanying changes during liposome infusion by monitoring pulsatile electrical impedance (rheoencephalogram- REG) on the skull (n = 24 pigs, 57 trials, 19 types of liposomes). A transient but significant decrease of REG pulse amplitudes followed the injection of liposomes (78.43% in the total sample, and 91.66% in the Doxil subgroup; P = 0.003, n = 12), indicating the involvement of cerebrovascular reaction during liposome infusion.

Keywords liposome, complement activation, cerebrovascular reactivity, rheoencephalogram

Introduction

Liposomal formulations of drugs, among them doxorubicin (Doxil), have been developed and increasingly used in the treatment of cancer (Anonymous, 2002; Gabizon and Muggia, 1998; Uziely, 1995; Wang et al., 2001). However, a common side effect after injection of liposomes is a poorly understood immediate hypersensitivity reaction in a

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relatively large percentage of patients (3–45%) (Laverman et al., 2001; Yee et al., 1998). The physiological signs of shock, including transient hemodynamic changes, hypotension, bradycardia, and cardiac arrhythmias, were shown recently to be reproducible in pigs by intravenous injection of minute amounts of liposomes including Doxil (Szebeni et al., 1999, 2000).

Following liposome infusion in humans, frequently reported neurological symptoms include tremor, nausea, dizziness, fainting, panic attacks, and imminent fear of death (Anonymous, 2002). These symptoms may be caused by complement-activation and subsequent release of anaphylatoxins C3a, C5a, which activate a secondary messenger system, mainly through C5a receptor bearing cells. The unleashing of a wave of secondary vasoactive mediators may involve cerebrovascular reaction, formation of thromboxanes, leukotrienes, platelet activating factor and histamine (Krause, 1999; Marceau et al., 1987; Moibenko et al., 1994). Based upon the above-mentioned facts, we hypothesized the involvement of cerebrovascular change during liposome infusion.

Cerebral blood flow (CBF) measurements were based on rheoencephalography (REG), an acknowledged but rarely used method to estimate CBF (Anonymous, 1997). Briefly described, the pulsatile electrical impedance reflects the change of electrical conductivity during the heart cycle because the blood and cerebrospinal fluid are better conductors than the dry tissue; therefore, the heart pumping function results in a change in electrical conductivity shown as a bio-impedance pulse wave. The bio-impedance pulse curve resembles a pressure pulse curve; its rising part is termed the anacrotic phase, and its decreasing part, the catacrotic phase. The pulsatile change is in the range of 0.1% to 1% of the total impedance (Patterson, 1995). When used on the head, this method is called rheoencephalography or REG (Jenkner, 1986). REG amplitude increase is a consequence of CBF and/or intracranial blood volume increase, reflecting vasodilatation, and REG amplitude decrease indicates CBF/blood volume decrease or vasoconstriction. Here we present REG data as a function of CBF autoregulation. The objective of this study was to describe the change of CBF/ blood volume during liposome infusion.

Methods

Animals

Administering of anesthesia and preparation of animals were detailed elsewhere (Szebeni et al., 2000). Briefly, pigs were anesthetized with 2% isoflurane. Inspired oxygen, and end tidal carbon dioxide (CO_2) tensions were maintained at 21 vol % and 35–40 mm Hg, respectively. Twenty-four male Yorkshire pigs were used (weighed 49.77 kg ± 28.25), and 19 types of liposomes were injected in 57 trials. A Doxil subgroup (n = 12) was also separately analyzed.

Liposomes

The liposomes were injected as intravenous bolus followed by 10 mL saline wash. Doxil (doxorubicin, Alsa Corp., Montain View, CA) was diluted in physiological saline, dosed usually at 10–50 μL.

CBF-REG

REG was measured by two stainless steel electrodes (5 × 20 mm) placed on the skull over the parietal and frontal bone of each pig using stainless steel screws. The anatomical

localization was determined by the atlas (Popesko, 1979). A Bovie gel conductor (Sybron, Rochester, NY) was layered between the bone and the electrode. The electrodes were placed apart from the sutura sagittalis symmetrically; the interelectrode distance was 12–35 mm. The electrodes were covered with dental acrylic cement (Cranioplastic Powder, Plastics One, Roanoke, VA). An electrical impedance amplifier (Rheographic Preamplifier KR, Carlo Erba/Galileo, Italy) was used (measuring frequency was 45 kHz). Basic interelectrode resistance was 3–5 ohms: this was the value read on a 10-turn potentiometer. This value involves both contact resistance and tissue resistance; it was not possible to measure them separately with this device.

Data Acquisition

Data acquisition and processing were done using PC-based A/D converter card (PC LPM 16) and BioBench data acquisition and analysis software (National Instruments, Austin, TX). Recorded modalities were as follow: electrocardiogram, systemic arterial pressure (SAP), pulmonary arterial pressure (PAP), REG, and respiratory CO_2. The A/D conversion sampling rate was 200 Hz. Data were recorded before and during liposome infusion and processed offline.

For data analysis, proprietary software (L. Baranyi) was employed. The REG and SAP tracings were first compared qualitatively and analyzed visually. In the Doxil subgroup REG pulse wave calculation and comparison were analyzed quantitatively, based on a REG amplitude measurement of 10-sec time-windows. A control segment of recording was chosen from the pretreatment period (baseline), and the changes were determined during drug infusion, where the REG pulse amplitude was minimal. In order to decrease the respiratory interference with REG, the data were digitally filtered (Butterworth bandpass, 0.3–60 Hz) with a software module integrated into the proprietary software. Values were expressed as mean ± SD.

There was no statistical treament for data on the 24 pigs (Table 1); only percentage values were calculated. All applied statistical test were used on the Doxil subgroup (Table 2). In this group averaging the differences of individual mean REG values would be misleading, as these differences are due to a different amplification of the original

Table 1
Summary of administered liposomes, their CBF effect, and SAP correlation

Pig	Liposome		CBF		Correlation with SAP		
	Total	Type	Increase	Decrease	AR	NAR	Mixed
24	57	19	19	40	27	25	15
	Percent of total		21.57	78.43	51.92	48.08	26.32

The CBF change was evaluated alone, and by its correlation to SAP: AR indicates that the change is autoregulatory; NAR indicates a non-autoregulatory relationship. If both types of changes were present within one trial, it was categorized as Mixed. CBF autoregulation was evaluated practically: if SAP and REG amplitude change were simultaneous but differed in direction. If decreases in REG, for example, coincided with SAP increases, we can state that the occurrence is a CBF autoregulatory response, and vice versa. The percentage calculation was performed within groups (100% was 51, the total number in CBF group, i.e., 11 + 40 = 51); and SAP correlation group (100% was 27 + 25 = 52). In mixed group total number of administered liposomes was considered as 100% (n = 57). Twenty-four male Yorkshire pigs were used and 19 types of liposomes were injected in 57 trials. Tracings with interfering artifacts were excluded from evaluation. The administered liposomes were detailed elsewhere (Szebeni et al., 2000, 2002).

Table 2
Average REG pulse amplitudes of baseline and of minimal values following Doxil administration

Pig ID	Baseline REG amplitude	Minimal REG amplitude	Difference %	Doxil mL/kg	AR	DC sec
43	0.19 ± 0.041	0.133 ± 0.034	70.00	0.92	+	–
47	0.292 ± 0.040	0.151 ± 0.080	51.58	0.29	+	194
50	3.091 ± 0.750	0.778 ± 0.424	25.16	1.70	+	54
54	1.938 ± 0.250	0.722 ± 0.454	37.27	1.38	+	80
55	123.4 ± 31.377	50.750 ± 17.669	41.13	1.34	+	175
64	18.143 ± 1.460	3.929 ± 1.492	21.65	0.51	+	–
65	81.714 ± 19.629	31.200 ± 17.925	38.18	0.61	–	177
66	42.4 ± 9.132	18.750 ± 14.436	44.22	0.58	+	21
67	2.924 ± 0.462	0.902 ± 0.326	30.85	0.37	+	–
68	2.194 ± 0.325	0.627 ± 0.178	28.59	0.46	+	106
73	0.112 ± 0.029	0.053 ± 0.014	47.05	0.59	+	75
74	0.248 ± 0.041	0.1848 ± 0.033	74.52	0.49	+	40
N = 12			42.52±16.51			

Mean REG pulse amplitude difference was 42.52 ± 16.51%; the group p-value was 0.003; n = 12; mean Doxil dose was 0.77 ± 0.46 mL/kg. In most cases (91.66%, 11 of 12) during a short SAP elevation REG amplitude decreased, indicated as autoregulatory response (AR +). Variations in individual mean REG values are due to different amplification of the original (analog) REG signal; averaging would be misleading. Time window of analysis was 10 sec. Column titles are as follows: Baseline: before Doxil administration; Minimal amplitude: during minimal REG amplitude values; Difference: percentage difference between baseline (control) and minimal REG amplitude following Doxil administration. REG amplitude values are arbitrary units, i.e., the measure of A/D. DC: Delay of CO_2 response showed two types of reaction: a rapid reaction (62.66 ± 30.51 seconds, n = 6) and a slower reaction (182 ± 10.44 seconds, n = 3). The statistical tests gave the same conclusion, specifically, that the means (or medians) were unequal ($P < 0.001$, $P = 0.03$). In two cases (pigs 64 and 67) there was no delay; in case of 47 CO_2 was not recorded. Data are mean ± SD.

(analog) REG signal; this is why we used nonparametric test and expressed the individual changes between baseline and Doxil administration in percentage and averaged these data resulting in a group mean.

Statistical tests were performed using Excel (Microsoft, Redmond, WA) and Minitab (Minitab Inc. State College, PA), respectively. The student t-test (assuming equal and unequal variances), Wilcoxon signed-rank test (for paired data, assuming equal and unequal variances) and Mann-Whitney test (test of equal medians) were used. A probability level of $P < 0.05$ was considered to be significant.

Results

The changes recorded following the liposome administration included a short (less than a minute long) increased SAP. This was followed shortly with a robust decrease, which was typically 60% of mean SAP or more. Simultaneously, a monotonous increase in PAP was observed. In unusually severe cases, pulmonary arterial pressure sometimes equaled systemic arterial pressure. The ECG recordings showed clear signs of arrhythmia and

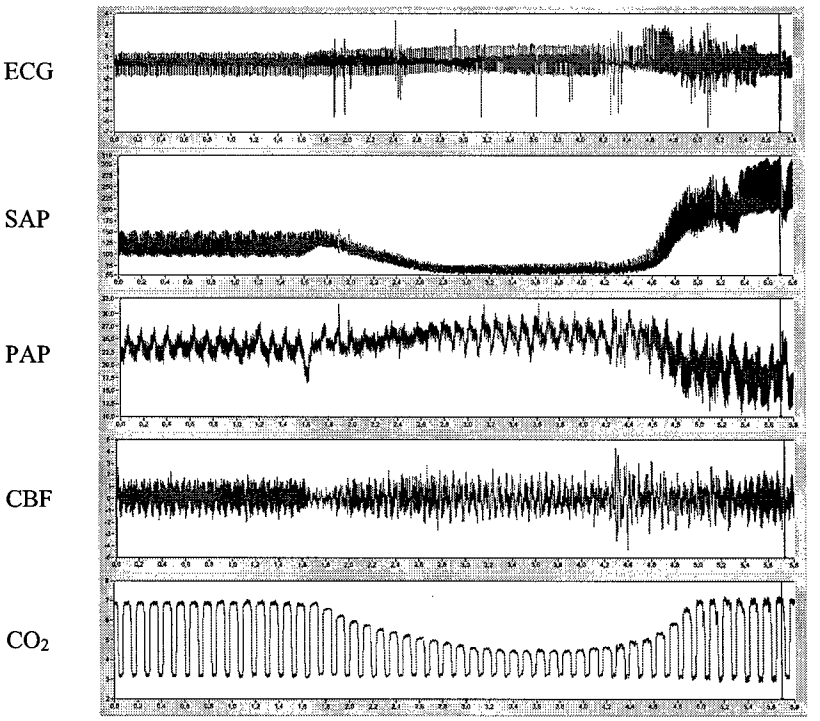

Figure 1. Typical reaction following Doxil administration (pig 68, dose 20 uL, i.v.). ECG showed bradycardia, arrhythmia, and signs of cardiac hypoxia/ischemia (ST depression, T amplitude increase); SAP decreased (baseline was 150/95, minimum was 77/55 mm Hg); PAP increased (baseline was 26/21, maximum was 29/23 mm Hg); and CO_2 decreased (baseline was 6.8, minimum was 4.4%). The CBF decrease was short (about 20 sec) (see enlarged area in Fig. 2) and preceded the decrease of SAP and CO_2 and the increase of PAP. Time window: 6 min; ECG: electrocardiogram; SAP: systemic arterial pressure; PAP: pulmonary artery pressure; CO_2: respiratory carbon dioxide concentration.

signs of cardiac hypoxia/ischemia, and bradycardia. The exhaled CO_2 decreased, indicating that the pulmonary gas exchange was strongly impaired due to pulmonary vasoconstriction and bronchospasm, as described earlier [Szebeni et al. (2000), Fig. 1]. Often, the animal could not recover from this event spontaneously, and resuscitation was needed. These signs of cardiovascular shock were associated with substantial changes in CBF (Fig. 2).

A significant decrease in REG pulse amplitudes was found in 40 of 51 cases. The amplitude decreased by 78.43% (Table 1). This transient decrease, which lasted only for a few seconds, preceded the actual onset of circulatory shock, before the pulmonary and systemic arterial pressures changed, and preceded the decrease of CO_2 levels in the exhaled air.

The decrease in REG pulse amplitude was followed by an increase, indicating transient cerebral vasoconstriction and vasodilatation. This occurred while SAP was low and even falling. However, only 51.9% of the animals followed this pattern. In the other group (48.1%), the pulse amplitudes remained depressed or decreased further; 26.3% of the animals showed both types of CBF response to cardiovascular shock induced by liposomes.

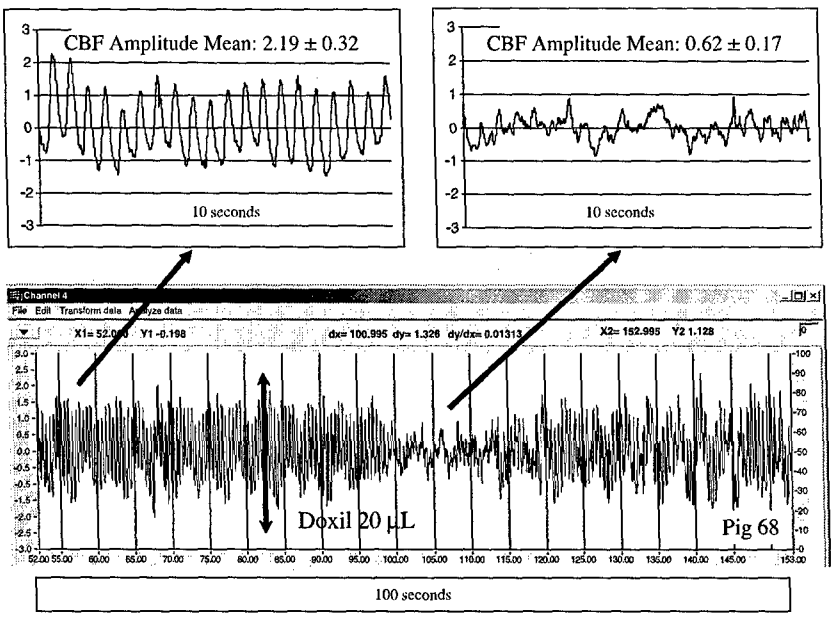

Figure 2. Typical CBF reaction following administration of Doxil (100-sec period, bottom tracing). Arrow indicates the administration of 20 uL Doxil (0.46 mL/kg). The window screen is from the applied DataLyser software. The upper tracings show two 10-sec magnified periods of the lower tracing (arrows indicate the origin). REG amplitudes are measured on these tracings. On all tracings, the Y-axis indicates volts, the measure of the analog digital converter. Results are mean ± SD. In cases with decreased REG pulses, the amplitude measurement was supported by other simultaneously recorded traces such as ECG (QRS complex) or the pulse of the systemic arterial blood pressure.

There was no qualitative difference in cardiovascular reactions to different liposomes; increased doses of all liposomes elicited severe reactions. Presented below is the result of a quantitative REG pulse amplitude analysis of a Doxil-treated group.

In the Doxil subgroup, the decrease in REG pulse amplitude was statistically significant ($P = 0.003$) after Doxil administration; average amplitude decrease was 42.5 ± 16.5%, and the CBF response was autoregulatory in 91.6% (11 out of 12) (Table 2).

The CBF autoregulatory response and CO_2 minimum showed two kinds of reactions: a rapid reaction and a slower reaction; the statistical tests supported the hypothesis of the existence of two types of reaction (Table 2).

Discussion

The use of liposome as a vehicle for drug administration is increasing; consequently knowledge of its side effects and ability to control its administration are desirable. One new, promising field for use of liposomes as a vehicle is in the treatment of cerebrovascular disease through gene therapy (Saito et al., 2004; Shi and Pardridge, 2000; Toyoda et al., 2003); another is for online monitoring of oxygen free radical production in the brain (Dirnagl et al., 1995).

This study gives the first direct evidence of cerebrovascular change during liposome administration. Although it was previously reported that liposome uptake

by capillary endothelial cells is nearly absent in the brain (McLean et al., 1997), the typical liposome injection study does not measure CBF (Mamot et al., 2003; Takada et al., 1982). Previous studies (Yoshimura et al., 2002) demonstrated a CBF increase following liposome infusion; however, the method used for measurement (laser Doppler flow with a probe) can measure only a very small volume (1.5 mm^3). Our method (REG) measures a much greater volume, the approximate volume of the entire brain. This methodological difference is important since it is known that CBF is heterogeneous; consequently, any local CBF cannot represent the total CBF; furthermore, local CBF measurement does not represent total CBF. Measurement of local CBF does not necessarily reflect CBF autoregulation (Bodo et al., 2004). Additionally, the Yoshimura study (Yoshimura et al., 2002) found that an increase in CBF may be a result of an unknown interaction with the infused materials (see CBF autoregulation discussion next). Liposome infusion requires further study to understand background clinical problems, such as brain blood barrier alteration (Johansson, 1992).

Hypersensitivity Reaction and Liposome

Animal studies of the cardiovascular effects of liposome tend to focus on the heart and carotid arteries used for cardiac catheterization (Pacher et al., 2003); these models are unsuitable for measuring CBF. Reports that attempt to describe the impact of liposome injection on CBF, especially under developing anaphylactoid shock, state that CBF was "scattered and patchy (Ekstrom-Jodal et al., 1982; Kapin et al., 1986; Miller et al., 1987; Parker and Emerson, 1977; Westerlind et al., 1991)." However, all agree that liposomes have considerable effects on CBF even without penetrating the blood-brain barrier, and they agree that changes in CBF can not be attributed to direct liposomal effects. The doxorubicin-induced free radical formation due to metabolic activation and its deleterious actions at the level of the membrane were previously described (Speth et al., 1988). Doxil exposure to endothelial cells and cardiomyocytes caused apoptotic cell death at sub-micromolar concentrations; the induced generation of H_2O_2 has been shown to be responsible for this drug's toxicity and apoptosis (Kalyanaraman et al., 2002). These facts may partially explain the observed CBF reaction: superoxide formation increased, nitric oxide production decreased; and peroxynitrite and hydrogen peroxide, which are potent oxidants implicated in several vascular pathologies, also increased (Vasquez-Vivar et al., 1997).

The observed CBF responses may at least partially result from complement activation and release of C5a anaphylatoxin and from vasoactive secondary mediators, small molecules able to penetrate the blood-brain barrier. These molecules may directly affect CBF autoregulation.

In these experiments a decrease in CBF was typical following liposome administration; however, individual reactions were different. This difference was caused by variations in liposome/Doxil dosage, the pig's sensitivity to the drug, and depth of anesthesia.

The described CBF reaction seems to be a mixture of two major types: one rapid, which was possibly mediated by myogenic mechanism of CBF autoregulation (Kontos et al., 1978), independent of the CO_2 level; the other reaction may be related to the thromboxane A2 and to other endothelial released factors, correlative with the CO_2 level. The role of the endothelium has been described in cerebral vasoconstriction (Krause, 2002). The cerebrovascular reaction described here, caused by liposome injection, is one example.

CBF Autoregulation

CBF autoregulation acts through vasomotor effectors that control cerebrovascular resistance (Chillon and Baumbach, 2002; Strandgaard and Paulson, 1984). It is possible to evaluate CBF autoregulation practically, that is, to record SAP and a CBF modality simultaneously to determine if changes are concurrent or not. If decreases in CBF, for example, coincide with SAP increases, we can conclude that the occurrence is a CBF autoregulatory response, and vice versa (Lang and Chesnut, 2002; Panerai, 1998).

According to a previous study (Chillon and Baumbach, 2002), CBF autoregulation in the cerebral circulation may be defined more pragmatically as the mechanism that protects the brain against the dangers of hypoxia at low perfusion pressures and against the risks of brain edema at high arterial pressures. Based on this definition, cerebral autoregulation may be thought of as a homeostatic mechanism that is superimposed over and above the baroreceptor reflexes. The baroreceptors, strategically located at the most proximal locations in the cerebral circulation, provide the first line of defense against acute ranges in arterial pressure. Autoregulation then serves as the next line of defense by helping to maintain constant cerebral capillary pressure, thus assuring a steady supply of essential metabolites and simultaneously protecting the blood-brain barrier. Several hypotheses (myogenic, neurogenic, and metabolic) have been proposed to account for the mechanisms that underlie autoregulation, detailed elsewhere. The anatomical background of CBF autoregulation concerns arterioles, the last small branches of the arterial system, which act as control valves through which blood is released into the capillaries. The arteriole has a strong muscular wall that is capable of closing the arteriole completely or of allowing it to be dilated several fold, thus having the capability of vastly altering blood flow to the capillaries in response to the needs of tissue (Ganong, 2001; Kontos et al., 1978). This arteriolar functioning can be visualized by functional MRI in brain imaging (Vainrub et al., 2004). Normally, CBF autoregulation is considered to be relatively independent of SAP, approximately between 50–150 mmHg. In other words, cerebrovascular reactivity works against SAP within this range. A routine test of cerebrovascular reactivity in clinical practice is administration of CO_2 inhalation, causing CBF increase. This test is used to evaluate the status of a patient during neurosurgery postoperative care. In such cases, nonreactive cerebrovascular vessels suggest a bad prognosis. The relationship of REG to CBF and its autoregulation was demonstrated during CO_2 inhalation, aortic compression, and hemorrhage (Bodo et al., 2003, 2004, 2005).

REG

Details of electrical impedance (bio-impedance) and REG measurements are given elsewhere (Geddes and Baker, 1989; International Conference on Bioelectrical Impedence, 1970; Jenkner, 1986). Various validation studies previously performed earlier established a correlation between CBF or flow, pressure, and electrical impedance (REG), detailed elsewhere (Bodo et al., 2001; Hadjiev, 1968; Jacquy et al., 1974; Jenkner, 1986).

One advantage of REG is its excellent time resolution. A drawback of REG is the inability to determine the exact source of the REG signal. Many factors appear to influence REG pulse formation under various physiological and pathological conditions, such as SAP, ICP, elasticity of arterial wall (Biophysical Aspects of Cerebral Circulation, 1980; Bodo et al., 1986). Early studies of REG do not address this problem. Therefore, we initiated studies to establish a correlation between known CBF changes investigating

its autoregulation using REG. The CBF modalities studied were 1) local CBF measured by laser Doppler flow; 2) carotid flow, measured by Doppler ultrasound; and 3) REG. The applied CBF manipulations were aortic compression (causing increased SAP and transient cerebral vasoconstriction), CO_2 inhalation, and hemorrhage (decrease of SAP causing transient cerebral vasodilation). All CBF manipulations confirmed that REG reflected classical CBF autoregulation (Bodo et al., 2003, 2004, 2005). These comparative CBF studies also demonstrated the differences between local CBF, global CBF, and carotid flow. It is known that cerebral arterial involvement in CBF autoregulation depends on the size of the vessel. The arteriola showed greater changes in diameter than larger arteries (Chillon and Baumbach, 2002; Kontos et al., 1978; Krause, 2002). Since REG showed CBF autoregulation, we conclude that REG reflects arteriolar functioning.

Conclusion

There is evidence that the complement–mediated reaction has a direct but transient impact on CBF. The fact that the CBF decrease preceded the CO_2 decrease during the reaction supports our hypothesis that liposome infusion has a direct impact on cerebrovascular reactivity. This observation has clinical significance in the administration of any liposome-encapsulated drug.

In summary, this study gives the first direct evidence of cerebrovascular changes occurring as a result of liposome administration. Further studies are needed to establish the impact of dose and size dependency of a liposome, e.g., Doxil (Speth et al., 1988) on CBF/blood volume changes and the potential overlapping of CBF autoregulation and direct vasoactive effect of complement activation (Szebeni et al., 2000, 2002).

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